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c-Jun/AP-1 transcription factor regulates laminin-1-induced neurite outgrowth in human bone marrow mesenchymal stem cells: Role of multiple signaling pathways

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ABSTRACT

In this study, we demonstrated that laminin-1 (LM-1) induces neurite outgrowth and enhances the expression of neurofilament-L and MAP2 in human bone marrow mesenchymal stem cells (MSCs). The c-Jun transcription factor was strongly activated by LM-1 during neurite induction. Suppression of c-Jun inhibited the expression of the c-Jun target genes α6 integrin and neurofilament-L, resulting in the loss of neurite outgrowth. Additionally, we found that the LM-1-α6 integrin interaction stimulated phosphorylation of FAK, leading to the activation of JNK and Akt. Pharmacological inhibition of these pathways blocked c-Jun activation and neurite outgrowth. Collectively, our findings suggest that c-Jun/AP-1 activity mediated by JNK, PI3K/Akt and ERK pathways is required for LM-1-induced neurite outgrowth in human bone marrow MSCs.

1. Introduction

Human bone marrow mesenchymal stem cells (MSCs) are a potential autologous source of stem cells for cell replacement therapy in neurodegenerative disorders. Transplantations of cells derived from MSCs have resulted in clinical improvement in animal models of neurological deficits [1,2]. Therefore efforts are being made to drive MSCs to a fate closer to neuron-like cells.

Extracellular matrix (ECM) proteins play essential roles in development, neurite outgrowth and differentiation. Purified laminin-1 (LM-1) and LM-1-derived peptides with the IKVAV sequence are potent inducers of neurite outgrowth in diverse neuronal cell types and primitive multipotent stem cells. LM-1 is reported to stimulate the expression of neurofilaments and voltage-gated sodium channels in unrestricted somatic stem cells derived from human umbilical cord blood [3]. A variety of multipotent stem cells treated with LM-1 or its derivatives displayed numerous neurite outgrowths in the absence of any growth factors [4–7].

The AP-1 protein, c-Jun transcription factor functions in axonal regeneration [8], neurite outgrowth [9,10] and neuronal differentiation [11]. c-Jun dimerises either with itself or with c-Fos, resulting in the transcription of several AP-1 responsive genes leading to diverse biological responses. c-Jun is also a prominent downstream nuclear target of ERK, JNK and PI3/Akt pathways [9,12]. In this study, we examined the role of c-Jun/AP-1 transcription factor in LM-1-induced neurite formation in human bone marrow MSCs. We demonstrate that c-Jun functions in LM-1-stimulated neurite outgrowth and is regulated by JNK, ERK and PI3K/Akt signaling. Also, our study identified integrin α6 and neurofilament-L as c-Jun regulated genes during the process of neurite outgrowth.

2. Materials and methods

2.1. Isolation and characterization of human bone marrow-derived mesenchymal stem cells

Previously, we have described the isolation and characterization of MSCs from human bone marrow [6]. Briefly, the bone marrow aspirates were obtained from adult donors according to the guidelines of ethics committees of National Centre for Cell Science and KEM Hospital, Pune. MSCs were isolated using Rosettesep MSCs Enrichment Cocktail (Stem Cell Technologies, Canada). The cells

Abbreviations: LM-1, laminin-1; PLL, poly-L-lysine; ASO, antisense oligonucleotides; SKI, Src kinase inhibitor-I

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were expanded in a complete culture medium (DMEM-low glucose with 10% fetal bovine serum).

2.2. Induction of neurite outgrowth in MSCs by LM-1

Culture dishes were coated with LM-1 and poly-L-lysine (PLL) (Sigma–Aldrich, MO) at 2.5 and 1 μg/cm^2, respectively, according to the manufacturer's instructions. MSCs cultured in complete culture medium were dislodged and washed with plain-DMEM to remove serum. The cells were then plated on PLL (used as a control) and LM-1-coated plates at 50% confluency in neurobasal/B27 medium (Invitrogen, CA). To visualize the morphological changes, the cells were fixed with 2% paraformaldehyde and stained with 0.3% Coomassie brilliant blue prepared in 10% acetic acid and 25% isopropanol. For quantitation of process outgrowth, 10–15 random fields consisting of 100–200 cells were photographed. The lengths of the cytoplasmic extensions were measured using Image Pro Plus software (version 5.0). Neurites were scored as processes greater than one cell diameter in length [13–15]. Neurite lengths were assessed in NF-L-positive and MAP2-positive cells on LM-1.

2.3. Integrin blocking experiments

MSCs were plated on LM-1-coated coverslips according to the procedure as described above, but the cells were incubated with function-blocking α6 integrin antibodies, azide-free (clone GoH3; Chemicon International, CA) used at 20 μg/ml. Control experiments were performed by plating MSCs on LM-1 in the presence of isotype-matched control antibodies of irrelevant specificity. The cells were incubated for 12 h before fixation and then proceeded for immnunocytochemical analysis.

2.4. Immunostaining

MSCs were cultured on PLL and LM-1-coated cover glasses for specified periods, fixed with 2% paraformaldehyde, and blocked with 10% goat serum. Next, the cells were incubated with appropriately diluted primary antibodies overnight at 4 °C. The antibodies used were neurofilament-L, MAP2, neurogenin-1, neuroD1 and α6 integrin, p-FAK (Tyr397) (all from Chemicon International, CA), GFAP (Thermo scientific) and c-Jun (Cell Signaling Technology, MA). The cells were washed and incubated with Cy3-conjugated secondary antibodies for 1 h. 4′,6-Diamidino-2-phenylindole (DAPI) (Invitrogen, CA) was used to stain the nuclei. The slides were visualized using a Nikon H600L Fluorescence Microscope System (Japan).

2.5. RNA isolation and semi-quantitative RT-PCR analysis

Total cellular RNA was extracted using TRIZOL® Reagent (Invitrogen) as per manufacturer's instructions. cDNA was prepared using Reverse Transcription Kit (Promega, Madison, WI 53711) and subsequently Taq DNA polymerase (Invitrogen, CA) was used to perform amplification of DNA. The parameters for a three-step PCR were 95 °C for 2 min, 1 cycle, then 95 °C for 15 s, 60 °C for 30 s, 72 °C for 60 s, 30 cycles. The primer sequences used were: forward c-Jun, 5′-CAGGTGGCACAGCTTAAACA-3′ and reverse c-Jun, 5′-TTTTTCTCTCC

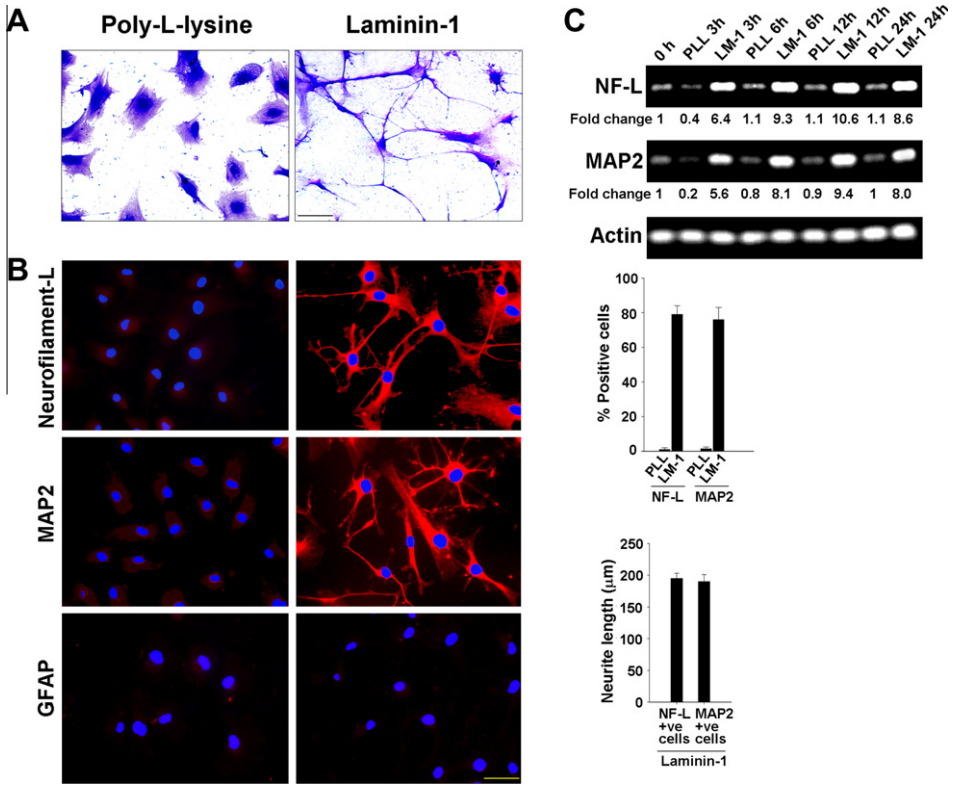

Fig. 1. Laminin-1-induced neurite outgrowth is accompanied by increased expression of neurofilament-L and MAP2 in MSCs. (A) MSCs were incubated on PLL and laminin-1-coated plates for 24 h, fixed, and stained with Coomassie brilliant blue for visualizing morphological changes (B) Cells grown on PLL and laminin-1-coated coverslips for 24 h were stained with anti-human neurofilament-L, MAP2, and GFAP antibodies followed by Cy3-conjugated secondary antibodies (red). Nuclei were labeled with DAPI (blue). Scale bar-100 μm. Percent cells positive for the expression of neurofilament-L (NF-L) and MAP2 on PLL and laminin-1 are shown in the bar diagram. Neurite lengths were calculated in NF-L-positive and MAP2-positive cells on laminin-1. Results are expressed as the mean ± S.E. of 100–200 cells counted in three independent experiments. (C) MSCs were treated with PLL or laminin-1 for the indicated time periods, and NF-L and MAP2 transcripts were examined by RT-PCR. The expression of NF-L and MAP2 was normalized to loading control (actin) and fold change over untreated cells (cells at 0 h point) is shown. The results are representative of three different experiments.

GTCGCAACT-3′; forward c-Fos, 5′-TACTACCACTCACCCGCAGA-3′ and reverse c-Fos, 5′-GTGGGAATGAAGTTGGCACT-3′; forward neurofilament-L, 5′-ACCCGACTCAGTTTCACCAG-3′ and reverse neurofilament-L, 5′-TTCCTCCACTTCGATCTGCT-3′; forward MAP2, 5′-AGGG CTGGTAGGTTGGATCT-3′ and reverse MAP2, 5′-ACTGGGTCACCAAA AAGCAC-3′; forward actin, 5′-AGCCTCTGATCTGTGCAGCG-3′ and reverse actin, 5′-TGACAGACCCGCAAGACAAA-3′. Quantification of PCR products was done by densitometry using the Image J software.

2.6. Antisense knockdown of c-Jun

Antisense oligonucleotides (ASO) were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the oligonucleotides were: 5′-GTTTCCATCTTTGCAGT-3′, antisense for c-Jun; 5′-ACCCTTGCGTCCGCTGCG-3′, scrambled for control. The antisense procedure has been previously described [16]. MSCs were plated on LM-1, and antisense and scrambled oligonucleotides were added directly to the culture media at 50 μM concentration. 12 h after plating, treatment of antisense and scramble oligonucleotides was repeated. Cells were monitored for neurite outgrowth and morphological changes for a period of 24 h.

2.7. Western blotting

MSCs were plated on LM-1 in the presence or absence of pharmacological inhibitors and harvested by scraping. Radioimmunoprecipitation assay (RIPA) buffer was used to prepare the whole cell extracts. Proteins in the lysates were resolved on 8–10% SDS–polyacrylamide gels, transblotted to PVDF membranes, blocked with 5% non-fat dried milk in Tris-buffered saline with 0.1% Tween-20, and probed with appropriately diluted primary antibodies against phosphorylated and total forms of c-Jun, FAK, Src, JNK and Akt (Cell Signaling Technology, Danvers, MA). The blots were incubated with HRP-conjugated secondary IgG antibodies (Biorad,

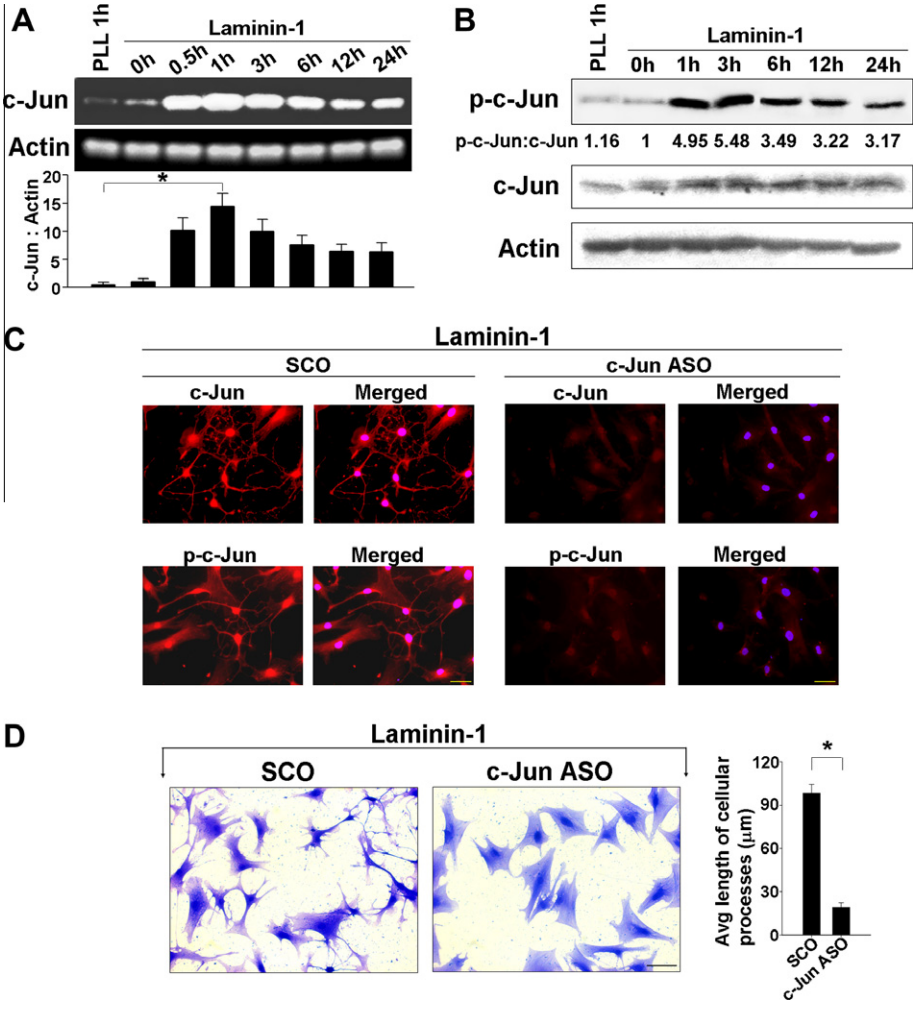

Fig. 2. c-Jun is involved in laminin-1-induced neurite outgrowth. (A) MSCs were cultured on laminin-1 for the indicated time periods and transcript levels of c-Jun were examined by RT-PCR analysis. c-Jun expression was normalized to loading control (actin) for each lane and fold change over untreated cells (cells at 0 h point) was calculated. The data shown is mean ± S.E. ($n = 3$) (*$P < 0.05$, PLL vs LM-1 treated for 1 h). (B) Kinetics of c-Jun phosphorylation (Ser63) was examined in laminin-1-treated cells by Western blotting. The amount of phospho-c-Jun was normalized to the total amount of c-Jun for each lane and fold change over untreated cells (cells at 0 h point) is shown. The blot is representative of two similar experiments. (C) MSCs were incubated on laminin-1 in the presence of scrambled oligonucleotides (SCO) and c-Jun antisense oligonucleotides (c-Jun ASO) for 24 h, fixed, and probed with antibodies against total and phosphorylated (Ser63) forms of c-Jun. Cy3-conjugated secondary antibodies (red) were applied to visualize the primary antibodies. Nuclei were labeled with DAPI (blue). Scale bar – 100 μm. The results are representative of three similar experiments. (D) Involvement of c-Jun in neurite formation was evaluated by plating MSCs on laminin-1 with scrambled oligonucleotides (SCO) and c-Jun antisense oligonucleotides (c-Jun ASO) for 24 h. The cells were later fixed and stained with coomassie brilliant blue. Scale bar – 100 μm. Average length of cellular processes from 100 to 200 cells of three different experiments was calculated and the results are expressed as mean ± S.E. (*$P < 0.05$).

CA) and immunoreactive bands were detected using Supersignal Femto chemiluminescent detection reagent (Pierce, IL). Quantification of protein bands was achieved by densitometry using the Image J software.

2.8. Statistical analysis

Data are expressed as mean ± S.E. Student's *t*-test was done using Sigma stat software (version 3.5); *P*-values < 0.05 were considered significant.

3. Results

3.1. LM-1-treated cells show increased expression of NF-L and MAP2

Human bone marrow-derived MSCs cultured on LM-1-coated plates for 24 h exhibited contracted cell bodies and prominent neurite-like extensions with 79 ± 5% and 76 ± 7% of cells staining positive for neurofilament-L (NF-L) and microtubule-associated protein 2 (MAP2), respectively (Fig. 1A and B). Approximately 40 ± 5% of the NF-L-stained cells and 38 ± 7% of the MAP2-stained cells displayed long cellular processes that were greater than one cell diameter in length and which complied with the criteria for neurites (see Section 2.2). The neurite lengths assessed using NF-L staining and MAP2-staining were comparable and were found to be 195 ± 8 μm and 190 ± 11 μm, respectively. RT-PCR analysis also showed that LM-1 significantly stimulated the expression of NF-L and MAP2 transcripts in MSCs (Fig. 1C). It was important to note that the cells plated on LM-1 did not show detectable expression of the glial cell-specific marker, glial fibrillary acidic protein (GFAP), neither at the protein level (Fig. 1B) nor at the transcript level (data not shown). Cells plated on PLL as controls did not show neurite outgrowth and displayed a fibroblastic morphology with negligible expression of NF-L and MAP2 (Fig. 1B). LM-1 also markedly stimulated expression of the proneural genes neurogenin-1 and neuroD1 during neurite induction (Supplementary Fig. 2). Process formation was evident on LM-1 till 72 h, which confirmed that neurite formation was a stable phenomenon (Supplementary Fig. 1). Robust stimulation of neurite outgrowth and increased expression of NF-L, MAP2 and proneural genes indicated that LM-1 modulates the expression of neuronal-specific genes in human bone marrow-derived MSCs.

3.2. LM-1 activates the c-Jun transcription factor during neurite induction

c-Jun is required for normal neurogenesis/neuronal development [8] and plays an important role in NGF-stimulated neurite outgrowth in PC12 cells [9,10]. In our study, LM-1 stimulated a rapid induction of c-Jun mRNA during neurite initiation. c-Jun transcript levels rose at 0.5 h (10.2-fold), peaked at 1 h (14.4-fold) and were maintained at a high level till 24 h (5.9-fold) (Fig. 2A). At the protein level, LM-1 induced a strong phosphorylation of c-Jun (ser63). Phosphorylation of c-Jun (ser63) was prominent at 1 h (4.95-fold), reached peak at 3 h (5.48-fold) and was evident till 24 h (3.17-fold). Total c-Jun levels remained fairly constant on LM-1, whereas cells at

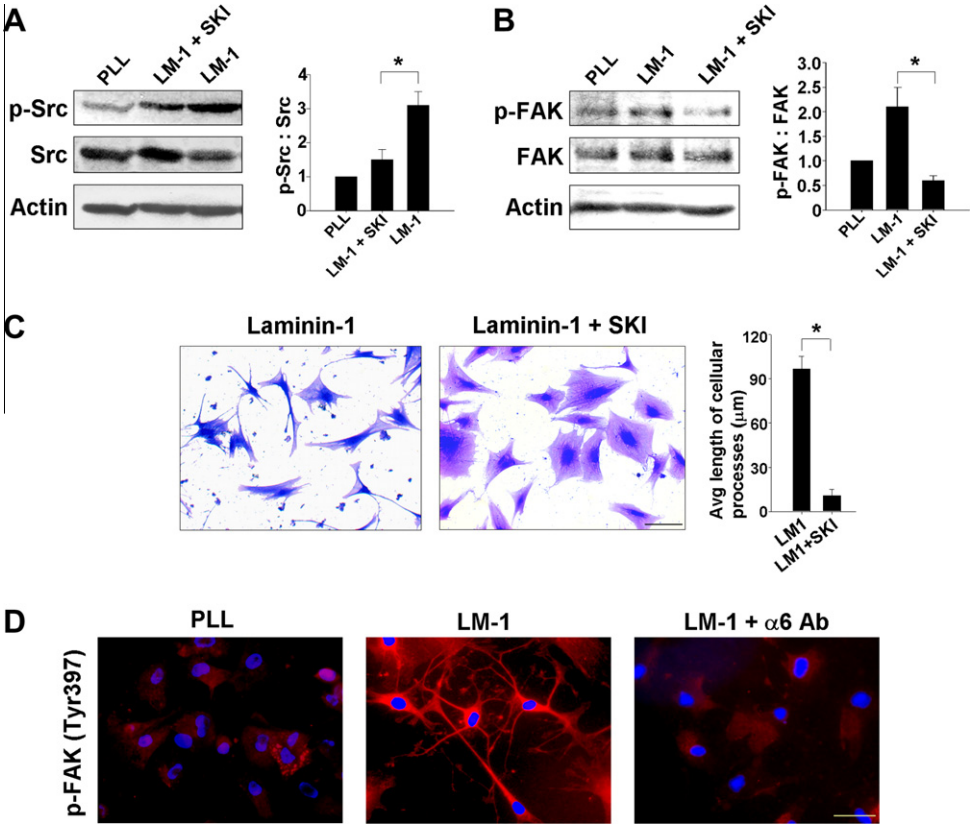

Fig. 3. FAK/Src signaling is necessary for neurite outgrowth. (A) and (B) Cell lysates were prepared from MSCs plated for 3 h on laminin-1 in the presence of Src kinase inhibitor-I (SKI) and were analyzed for phosphorylated and total forms of Src and FAK. The levels of phospho-proteins were normalized to the levels of total proteins and fold change over PLL treated cells is shown. Results are expressed as the mean ± S.E. of three independent experiments (*$P < 0.05$). (C) MSCs were plated on laminin-1 in the presence or absence of Src kinase inhibitor-I (SKI) for 24 h and assayed for neurite formation. Scale bar – 100 μm. Average length of cellular processes from at least 100 cells of three different experiments was calculated and the results are expressed as mean ± S.E. (*$P < 0.05$). (D) MSCs were plated on laminin-1-coated coverslips for 12 h in the presence or absence of function blocking of antibodies against α6 integrin (20 μg/ml). The cells were fixed and probed with anti-p-FAK (Tyr397) antibodies, followed by labeling with Cy3-conjugated secondary Ab (red). Nuclei were labeled with DAPI (blue). Scale bar – 100 μm.

the 0 h time point and cells plated on PLL showed low levels of phosphorylated as well as total c-Jun (Fig. 2B).

As c-Jun dimerises with c-Fos while affecting various biological responses, we investigated whether LM-1 stimulated the expression of c-Fos during neurite induction. Kinetic analysis of c-Fos transcript levels post-LM-1 attachment revealed that c-Fos mRNAs were significantly elevated at 0.5 h (7.3-fold), peaked at 3 h (12.1-fold) and were clearly detectable even at later time points. As with c-Jun, c-Fos expression was found to be low in cells at the 0 h point and in cells plated on PLL (Supplementary Fig. 3).

3.3. c-Jun expression is necessary for LM-1-induced neurite formation

The role of c-Jun in LM-1-induced neuritogenesis was evaluated by antisense suppression of c-Jun. The efficiency of the antisense procedure was tested by immunofluorescence examination of c-Jun expression. Transfection of c-Jun-specific ASO but not scrambled oligonucleotides (SCO) reduced the expression of c-Jun significantly in LM-1-treated cells (Fig. 2C). Also, the antisense-mediated depletion of c-Jun produced a prominent decrease in the level of phosphorylated-c-Jun (Fig. 2C) and markedly inhibited neurite outgrowth in cells plated on LM-1 (Fig. 2D). Together, these findings suggest that c-Jun is required for LM-1-stimulated neurite formation in MSCs.

3.4. FAK/Src signaling is essential for neurite formation on LM-1

ECM-integrin interaction activates non-receptor protein tyrosine kinases such as FAK and Src [17]. Recent studies have shown that Src phosphorylates several sites on FAK, resulting in the enhancement of FAK activity and recruitment of proteins such as Grb2 and PI3K for initiating downstream signaling events [18]. To examine whether FAK activation on LM-1 is dependent on Src activity, MSCs were plated on LM-1 in the presence of Src-specific inhibitor, Src kinase inhibitor-I (SKI) (15 μM) and were assessed for phosphorylation of both Src (at Tyr416) and FAK (at Tyr397). SKI blocked phosphorylation of Src as well as FAK, by 60 ± 4% and 62 ± 6%, respectively (Fig. 3A and B) and abolished neurite formation on LM-1 (Fig. 3C). Altogether, we conclude that Src activity is required for FAK activation, and that inhibition of FAK/Src signaling by SKI abrogates LM-1-induced neuritogenesis.

3.5. Function-blocking of α6 integrin inhibits phosphorylation of FAK and neurite formation on LM-1

Integrin signaling stimulated by ECM is primarily mediated through FAK activation [17,19]. α6β1 integrin is the major receptor for LM-1 implicated in neurite formation [20]. In our study, MSCs cultured on LM-1 in the presence of α6 integrin function-blocking

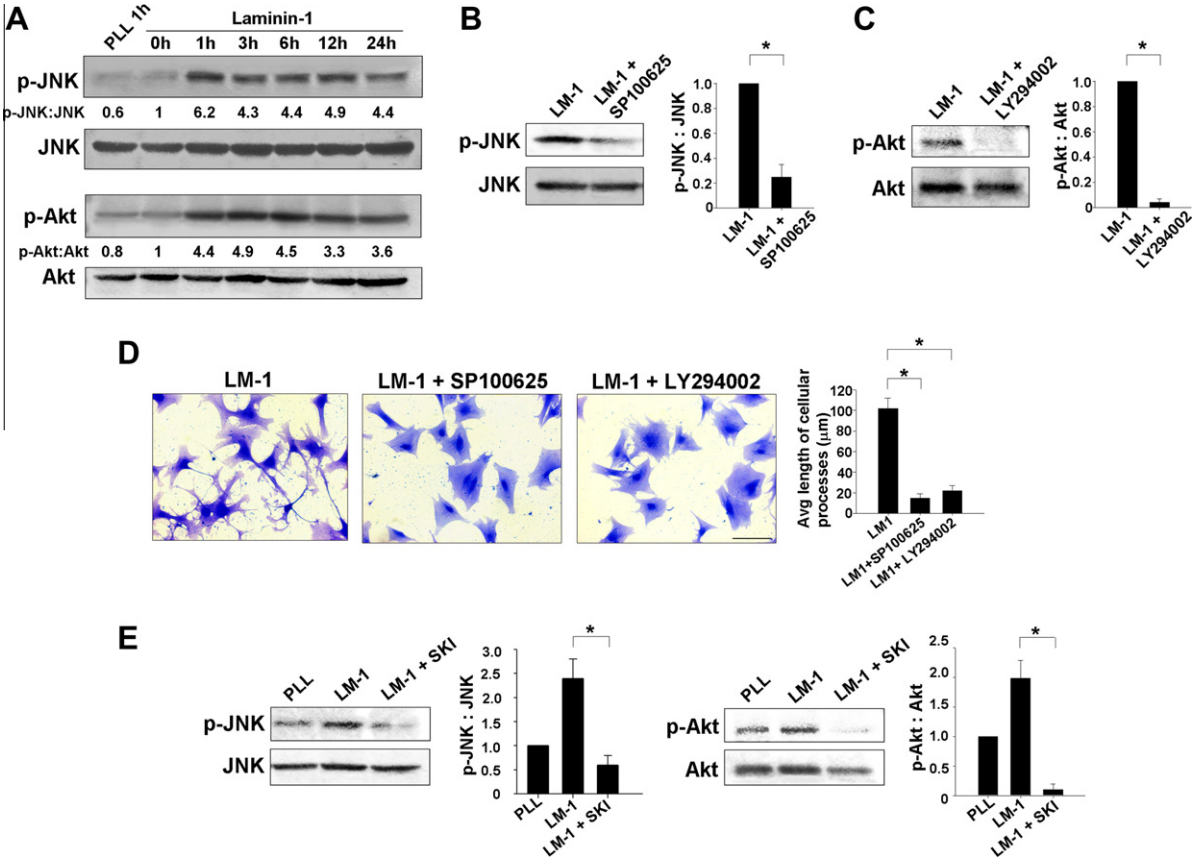

Fig. 4. Akt and JNK play a crucial role in laminin-1-stimulated neurite outgrowth (A) MSCs were plated on laminin-1 for the indicated time periods and protein samples were analyzed by Western blotting for phosphorylated and total forms of JNK and Akt. The amounts of phospho-proteins were normalized to the amounts of total proteins and fold change over untreated cells (cells at 0 h point) is shown. (B) and (C) MSCs were plated on laminin-1 in the presence of SP100625 and LY294002 for 3 h and examined for the activation of JNK and Akt, respectively. Bands were quantified by densitometry and the results are expressed as the mean ± S.E. of three independent experiments (*$P < 0.05$). (D) Functionality of JNK and Akt in laminin-1-induced neurite outgrowth was assessed by treating cells with SP100625 and LY294002, respectively. Cells were monitored for neurite formation for 24 h. Scale bar – 100 μm. Average length of cellular processes from 100 to 200 cells of three different experiments was calculated and the results are expressed as mean ± S.E. (*$P < 0.05$). (E) MSCs were plated on laminin-1 with Src kinase inhibitor-I (SKI) for 3 h and examined by Western blotting for p-JNK and p-Akt levels. The levels of phospho-proteins were normalized to the levels of total proteins and fold change over PLL treated cells is shown. Results are expressed as the mean ± S.E. of three independent experiments (*$P < 0.05$).

antibodies showed marked reduction in p-FAK (Tyr397) and loss of neurite outgrowth (Fig. 3D). These findings demonstrated that α6 integrin is involved in the activation of FAK and neurite formation on LM-1.

3.6. JNK and Akt pathways are involved in LM-1-induced neurite outgrowth

FAK/Src signaling activates several signaling cascades such as Ras-Raf-ERK, PI3K/Akt and SAPK/JNK pathways [17,18]. Previously, we had shown that FAK-mediated ERK activation was essential for LM-1-induced neurite outgrowth in MSCs [6]. In the current study, we investigated the involvement of JNK and Akt in neurite formation. As shown in Fig. 4A, LM-1 induced a rapid phosphorylation of JNK and Akt in MSCs, and activities of these kinases were maintained at a high level on LM-1 even at later time points. Phosphorylation of JNK and Akt was found to be low in cells at 0 h point and in cells plated on PLL. Treatment with JNK inhibitor-SP100625 or Akt inhibitor-LY294002 reduced levels of p-JNK by 65 ± 4% and p-Akt by 93 ± 3%, respectively (Fig. 4B and C) and blocked neurite formation on LM-1 (Fig. 4D). We also examined further whether FAK/Src signaling controlled the activation of JNK and Akt. Interestingly, SKI treatment significantly reduced the levels of p-JNK by 70 ± 5% and p-Akt by 92 ± 3%, confirming that these kinases are regulated by FAK/Src signaling (Fig. 4E).

3.7. c-Jun activation is regulated by FAK/Src, MAPK and PI3K/Akt signaling

Since LM-1-induced neurite outgrowth involved activation of FAK as well as c-Jun, it was important to examine whether c-Jun is regulated by FAK/Src signaling. Treatment with SKI reduced phosphorylation of c-Jun by 69.89%, confirming that c-Jun activation is regulated by FAK/Src signaling (Fig. 5A). Recent studies have shown that MAPK and PI3K/Akt pathways regulate the activation of c-Jun [9,12]. Therefore, we analyzed the effect of SP100625 (JNK inhibitor), LY294002 (PI3K/Akt inhibitor), U0126 (MEK inhibitor) and PD98059 (ERK inhibitor) on the activation and expression of c-Jun. As shown in Fig. 5B, the amount of phosphorylated c-Jun was normalized to the total amount of c-Jun for each lane and fold phosphorylation was calculated. LY294002 and SP100625 treatments inhibited LM-1-stimulated phosphorylation of c-Jun by 60.23% and 63.74%, respectively. U0126 and PD98059 reduced the activation of c-Jun on LM-1 by 59.06% and 56.72%, respectively. It was important to note that U0126 and PD98059 decreased the levels of total c-Jun protein substantially, indicating that MEK–ERK signaling also controlled the expression of c-Jun (Fig. 5B).

3.8. LM-1-stimulated expression of α6 integrin and neurofilament-L is regulated by c-Jun

α6 integrin and neurofilament-L, the proteins involved in neurite outgrowth, are known target genes of c-Jun [21–24]. As shown in Fig. 5C, during neurite induction, LM-1 enhanced the expression of neurofilament-L and α6 integrin. We examined whether c-Jun regulated the expression of α6 integrin and neurofilament-L during neurite formation. Transfection of c-Jun ASO significantly inhibited the expression of α6 integrin and neurofilament-L on LM-1, resulting in the loss of neurite outgrowth (Fig. 5C). These findings confirmed that c-Jun regulates the expression of α6 integrin and neurofilament-L during neurite formation.

4. Discussion

Purified LM-1 and LM-1 derived peptides have neurite-promoting activity in a wide variety of neural cell types and primitive stem

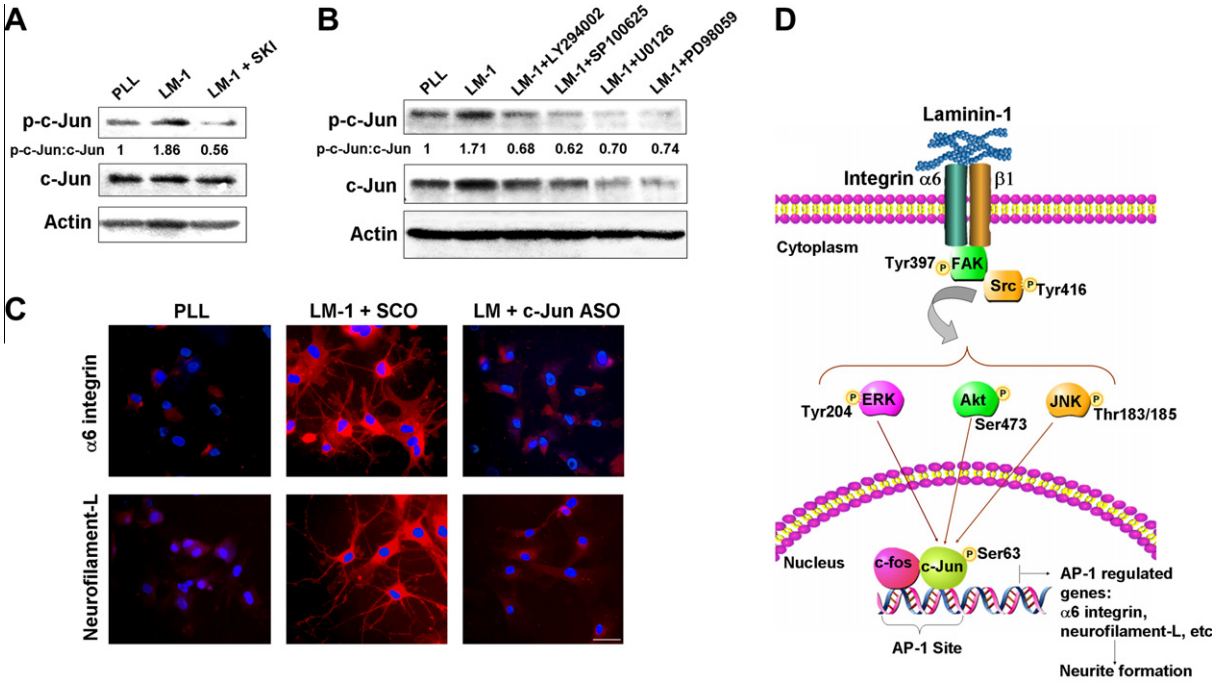

Fig. 5. Role of c-Jun in the regulation of neurite outgrowth. (A) MSCs were plated on laminin-1 with Src kinase inhibitor-I (SKI) for 3 h and analyzed by Western blot for p-c-Jun (ser63) (B) MSCs were incubated on laminin-1 with LY294002, SP100625, U0126 and PD98059 for 6 h and cell lysates were analyzed by Western blot for phosphorylated c-Jun (ser63) and total c-Jun. PLL-coated plate served as a control. Band intensities were quantified by densitometry. The amount of phospho-c-Jun was normalized to total amount of c-Jun for each lane and fold change over PLL-treated cells is shown. The blot is representative of two similar experiments. (C) MSCs were incubated on laminin-1 in the presence of c-Jun antisense oligonucleotides (c-Jun ASO) and scrambled oligonucleotides (SCO) for 24 h. The cells were later fixed and probed with α6 integrin and neurofilament-L antibodies followed by labeling with Cy3-conjugated secondary Ab (red). Nuclei were labeled with DAPI (blue). Scale bar – 100 μm. (D) Proposed mechanism for the regulation of c-Jun activity by different kinases and for the role of c-Jun in laminin-1-induced neurite outgrowth in MSCs.

cells. This study aimed to decipher the molecular mechanisms underlying neurite outgrowth stimulated by LM-1 in human bone marrow MSCs. Here, we demonstrated for the first time that the AP-1 protein, c-Jun transcription factor is involved in LM-1-induced neurite outgrowth in MSCs. The present report also showed that c-Jun activity is regulated by JNK, ERK and PI3K/Akt signaling pathways.

In a recent study in unrestricted somatic stem cells (USSCs) from human umbilical cord blood, LM-1 stimulated the expression of neurofilament mRNAs and induced neurite-like extensions. However, dopamine secretion was observed in USSCs only after the supplementation of a cocktail of growth factors [3]. LM-1 nanofibers had stimulated development of elaborate neurite-like extensions in human adipose stem cells (ASCs) in serum-free media without differentiation factors [4]. In our study, LM-1-induced neurite outgrowth was accompanied by increased expression of NF-L, MAP2, and proneural genes, but not GFAP, indicating that LM-1 modulates the expression of neuronal-specific genes in human bone marrow MSCs. Several studies have employed "chemical stimulators" for the transdifferentiation of MSCs into neuron-like cells [25,26]. There is a great deal of controversy about these studies, as some reports show that the morphological changes such as neuritic processes after "chemical induction" are likely the result of cellular toxicity and cell shrinkage [27,28]. In this study, we have used a natural ECM protein, LM-1, which was free from toxic effects on MSCs. c-Jun is reported to play a crucial role in nerve growth factor (NGF)-dependent neuronal differentiation of SH-SY5Y cells [11]. In PC12 cells, LM-1 stimulated phosphorylation of c-Jun prominently during neurite induction [29] and siRNA-mediated downregulation of c-Jun abolished NGF-induced neuritogenesis [9]. Consistent with these reports, we found that LM-1 stimulated a sustained phosphorylation of c-Jun during neurite induction and transfection with c-Jun-specific ASO blocked LM-1-induced neurite outgrowth in MSCs.

Integrin-mediated FAK/Src signaling was found to be crucial for LM-1-stimulated neurite outgrowth in adult DRG neurons [30] and in embryonal cortical neurons [31]. In this study, we showed that FAK/Src signaling regulated LM-1-stimulated neurite outgrowth in MSCs. The FAK–Src complex may activate the kinases ERK, Akt, and JNK, the signaling molecules known to play key roles in neurite outgrowth. In NGF-treated PC12 cells, JNK and ERK pathways effected neurite formation by converging on the level of target proteins such as c-Jun [32]. Consistent with this report, we also found that JNK, Akt and ERK pathways converged on c-Jun activation during neurite outgrowth in MSCs. In a recent report in PC12 cells, multiple pathways (JNK, ERK and P38) were reported to regulate c-Jun activity and neurite outgrowth [9]. In our study, pharmacological inhibition of either JNK or Akt significantly blocked c-Jun activation and completely abrogated neurite outgrowth stimulated by LM-1. The total loss of neurite outgrowth may be attributed to the low magnitudes of c-Jun activation in these instances. In SP100625 and LY294002 treated cells, the phospho-c-Jun level was prominently reduced and was closer to levels seen in PLL-treated cells. But we had already seen that a low level of phosphorylated-c-Jun, as observed in cells plated on PLL, does not result in neurite formation. Hence, it appeared that a certain threshold of c-Jun activation is a prerequisite for neurite formation to take place. High levels of phospho-c-Jun, as seen in cells plated on LM-1 (without inhibitors), seem to be necessary for the stimulation of neuritic processes.

Activation of c-Jun results in the transcription of its target genes such as α6 integrin [21,22], neurofilament-L [23,24], CD44 and galanin [8], the molecules known to be involved in neurite outgrowth. In our study, antisense suppression of c-Jun attenuated expression of α6 integrin and neurofilament-L during neurite formation, confirming that these genes are regulated by c-Jun.

5. Conclusions

Based on our findings, we propose a model that shows the regulation of c-Jun activity by multiple signaling pathways and which depicts the role of c-Jun/AP-1 transcription factor in LM-1-stimulated neurite formation. LM-1-α6 integrin interaction stimulates FAK/Src signaling leading to the activation of ERK, JNK, and Akt. These kinase pathways converge on c-Jun activation, leading to the expression of c-Jun target genes (α6 integrin and neurofilament-L) and neurite outgrowth in human bone marrow MSCs (Fig. 5D).

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.04.072.

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